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Short communication

Mechanisms of arachidonic acid induced glial swelling

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Abstract

Accumulation of arachidonic acid (AA) in the brain during ischaemia may contribute to development of brain oedema. In this study we investigated the effect of selected drugs on AA-induced cytotoxic brain oedema in C6 glioma cells. Suspended C6 glioma cells were preincubated with drugs and AA (0.1 mM) was added. When no drug was administered cell volume increased immediately after the addition of AA with a maximum cell swelling of 13.1 ± 1.9% at 15 min (mean ± S.E.M.). Preincubation of cells with BW 755C, a dual inhibitor of cyclo- and lipoxygenases, showed no reduction in cell swelling from AA, whereas superoxide dismutase, amiloride and the protein kinase inhibitor H-9370 led to a significant attenuation of volume increase ($p < 0.05$). The role of Na^+ ions during cell swelling from AA was evaluated after pretreatment of C6 glioma cells with ouabain. This resulted in a reversal of cell swelling ($p < 0.01$). We conclude that there is potential involvement of free radicals, signal transduction systems and intracellular accumulation of Na^+ ions in glial cell swelling from AA.

Keywords: Arachidonic acid; Cytotoxic brain oedema; Cerebral ischaemia; In vitro model

The physiological concentration of free arachidonic acid (AA) in brain tissue is very low (< 0.01 mmol/kg) [8]. Ischaemia may contribute to the accumulation of free AA in brain tissue through the activation of phospholipases (A_2, C). The subsequent release of AA may lead to a rise in AA concentration of up to 0.5 mmol/kg [16].

Increased cerebral concentrations of AA trigger a cascade of pathophysiological events. Thus, when added to brain slices in vitro AA induced brain oedema [2]. Recent results have also shown that the incubation of astrocytes from primary culture or C6 glioma cells with AA led to cell swelling in a dose-dependent manner [19].

The noxious effects of AA, which may contribute to cerebral oedema, include enhanced production of eicosanoids, especially that of prostaglandins and leukotrienes [20], as well as superoxide anion radicals [3], both of which have been shown to play a crucial role in the pathogenesis of brain oedema and cell injury in vivo [1,2,5]. Furthermore, AA causes intracellular acidosis, probably due to mitochondrial dysfunction [19]. Elevated concentrations of protons might accelerate formation of other oxygen-derived radicals, such as peroxyl radicals (OOH) which are potent initiators of lipid peroxidation [18]. This radical-induced chain reaction causes disruption of membrane integrity. Enzymes which are important for the maintenance of cell volume, i.e., Na^+/K^+-ATPase, are dislocated [14]. This process may lead to cell swelling and eventually to cell death [19].

The above described pathophysiological events following rapid accumulation of AA may be responsible for oedema development secondary to cerebral ischaemia, thus representing targets of possible pharmacological intervention in order to prevent further damage.

The aim of the present study therefore was to examine the effect of various pharmacological substances on AA-induced glial swelling in vitro using C6 glioma cells.

Cells were grown, subcultivated and harvested according to standard procedures [19,20], and were incubated in a specially designed plexiglas incubation chamber [11,19,20].

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Cell volume was determined by flow cytometry using the advanced Coulter system with hydrodynamic focusing (HEKA-Elektronik, Lambrecht/Pfalz, Germany), which is able to detect changes in cell size of ~1% [9].

Each experiment was preceded by a control period of 45 min in order to obtain constant cell volume, viability, cell density and medium osmolality. The average of the last three cell-volume measurements during the control period was taken as the reference for the cell volume of the subsequent experimental phase, which was started by injection of AA into the suspension medium (final concentration in cell suspension 0.1 mM). Cell volume was recorded during 90 min. The effect of AA, given as sodium arachidonate, on cell volume was evaluated in a separate group ($n = 4$).

In the other groups, cells were pretreated with different drugs. BW 755C (0.2 mM [6]), a dual inhibitor of cyclo- and lipoxygenases, and superoxide dismutase (SOD; 300 U/ml) were used to evaluate the significance of fatty acid metabolites and superoxide radicals in the AA-induced swelling process ($n = 4$–5). Amiloride (0.1 mM [13]), an inhibitor of the Na^+/H^+-antiporter, and a protein kinase C (PKC)-inhibitor (H-9370; 0.001 mM [7]) were chosen to investigate the importance of the Na^+/H^+-antiporter in glial swelling from AA ($n = 4$–5). Finally, C6 glioma cells were preincubated with ouabain (1.0 mM [13]), an inhibitor of the Na^+/K^+-ATPase, in order to analyse the role of Na^+ ions in the swelling process. Each drug was added 15 min before AA was injected into the medium, except for ouabain, which was given 45 min prior to the beginning of the experimental phase. The effect of each drug on cell volume was tested in separate control groups ($n = 3$–10) in which the drug alone without AA was added to the cell suspension.

The results are shown as mean ± standard error of mean (S.E.M.). The cell volume after pretreatment with the abovementioned drugs was compared to that when AA alone was added to the cell suspension. The difference in cell volume was analysed for statistical significance using the Kruskal–Wallis test for nonparametric one-way analysis of variance and multiple comparisons of rank for independent samples.

Incubation of C6 glioma cells (pH = 7.4; pO_2 = 80–100 mmHg; 37°C) without addition of AA showed a constant cell volume over 90 min (Figs. 1–3). Cell swelling occurred immediately after injection of AA (0.1 mM). Changes in cell volume were compared to the mean cell volume of the last three control measurements (794.0 ± 18.8 μm^3). The volume increased to 113.1 ± 2.5% of control after 15 min ($p < 0.001$), thereafter it decreased and reached 109.2 ± 2.5% ($p < 0.001$) of control after 90 min (Figs. 1–3).

When C6 glioma cells were pretreated with BW 755C there was no statistically significant difference in volume increase compared to the group in which AA alone was administered (Fig. 1). Preincubation of the cell suspension with superoxide dismutase showed a significant volume decrease between 40 and 70 min ($p < 0.05$, Fig. 1) after an initial increase in cell volume during the first 20 min.

Pretreatment of C6 glioma cells with amiloride and with the PKC inhibitor H-9370 resulted in cell swelling during the first 7 and 20 min, respectively. Thereafter the volume

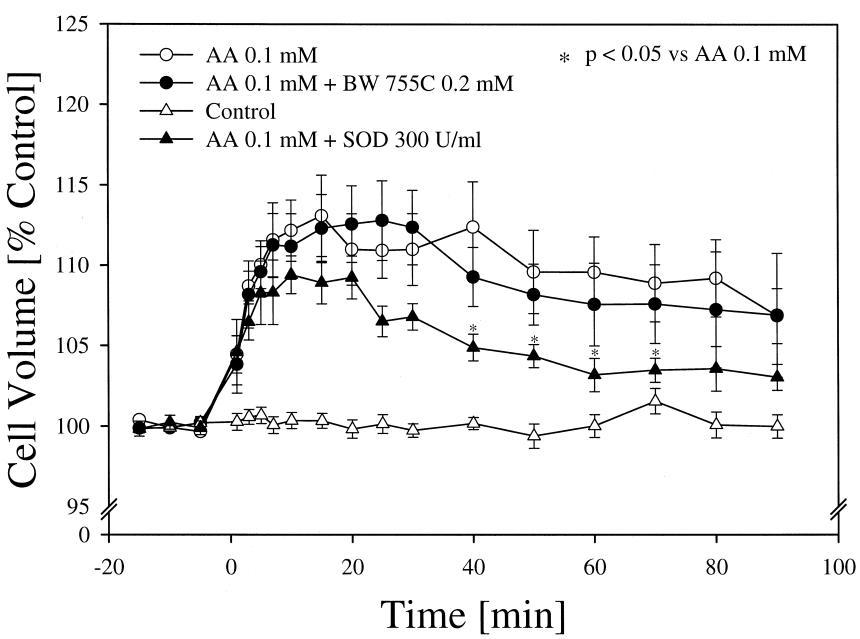

Fig. 1. Volume response of C6 glioma cells incubated with AA after pretreatment of cells with the cyclo- and lipoxygenases inhibitor BW 755C or the superoxide dismutase inhibitor SOD. Filled symbols indicate the presence of the drug *and* AA in the suspension medium; open symbols represent incubation of cells with AA alone as well as incubation of cells without addition of drugs. There was no significant difference in AA-induced cell swelling between incubation of C6 glioma cells with AA alone and pretreatment of cells with BW 755C, whereas SOD reduced cell swelling significantly.

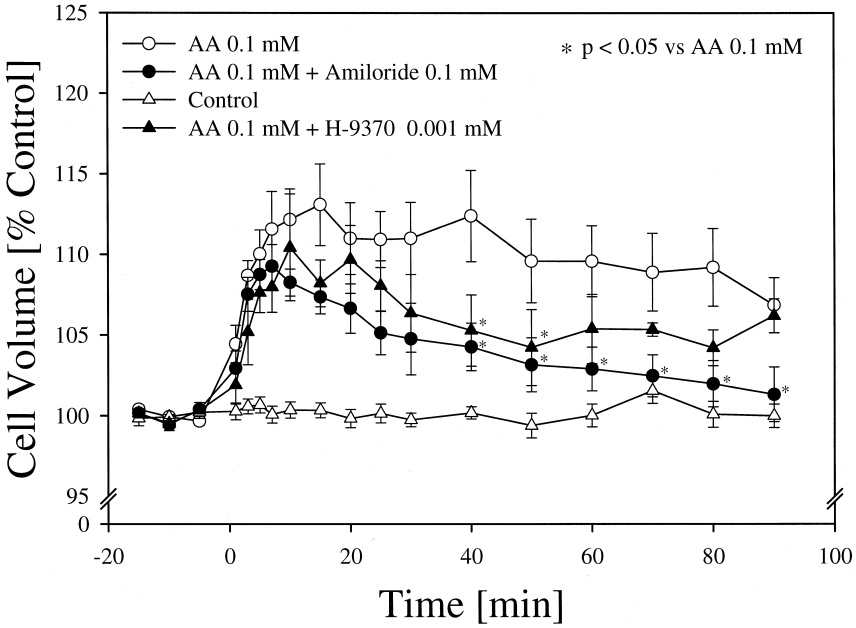

Fig. 2. Volume response of C6 glioma cells incubated with AA and pretreated with the Na^+/H^+-antiporter inhibitor amiloride or a competitive inhibitor of PKC (H-9370). The reduction in cell swelling by amiloride and the PKC inhibitor was similar and reached significance in both cases.

decreased continuously, reaching significance as indicated in Fig. 2 ($p < 0.05$, Fig. 2).

Preincubation with ouabain prevented glial swelling from AA almost completely ($p < 0.01$; Fig. 3). A slight initial volume increase with the maximum cell volume being $106.0 \pm 1.1\%$ of control was followed by a steady volume decrease, with control values being regained after 20 min incubation with AA (Fig. 3).

Incubation of C6 glioma cells with BW 755C, SOD, amiloride, H-9370 and ouabain without subsequent addition of AA did not lead to significant cell swelling (not shown, Fig. 3).

C6 glioma cells were used in our experiments because of their homogenous volume allowing accurate quantification of even slight volume changes that can be detected by a well-established in vitro model. This enables us to

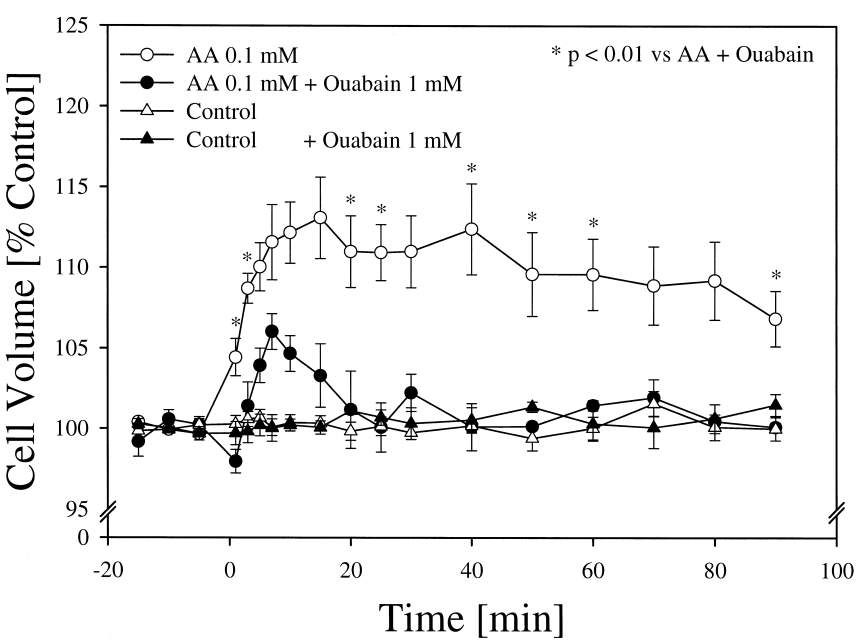

Fig. 3. Volume response of C6 glioma cells after addition of AA and pretreatment of cells with ouabain, an inhibitor of the Na^+/K^+-ATPase, for 45 min. Ouabain prevented AA-induced cell swelling by increasing the intracellular Na^+ concentration leading to a reduction in the extra-/intracellular Na^+-gradient.

observe single pathomechanisms under otherwise constant extracellular conditions [11,19]. Furthermore, C6 glioma cells have many glial specific properties which are comparable to those of astrocytes from primary culture, including marker proteins (S-100 and glial fibrillary acidic protein) as well as enzymes, ion carriers and uptake systems for neurotransmitters [12].

C6 glioma cells and astrocytes from primary culture are both known to produce prostaglandins and leukotrienes [20]. Enzymes, such as Na^+/K^+-ATPase and PKC are present in both cell lines and exhibit similar activities [10,12,15]. In both cell types the concentration of superoxide anions was found to be the same after 30 min incubation with AA [3,4]. Furthermore, the Na^+/H^+-antiporter is present in C6 glioma cells and astrocytes from primary cultures [12,13].

Pathophysiological conditions such as ischaemia can lead to an increase in free AA [16], a substrate for cyclo- and lipoxygenases. This may result in an accumulation of eicosanoids, such as prostaglandins and leukotrienes in the brain which might contribute to the development of cytotoxic and vasogenic brain oedema, postischaemic hypoperfusion, as well as irreversible neuronal damage [1,2,5,19]. In this study, pretreatment of C6 glioma cells with BW 755 C did not reduce cell swelling from AA (Fig. 1), although in previous experiments the inhibitor was shown to reduce the production of LTB_4 and $PGF_{2\alpha}$ by C6 glioma cells after incubation with 0.1 mM AA [20]. These results suggest that AA itself and not eicosanoids might be responsible for cytotoxic glial swelling from AA.

In addition to the production of eicosanoids, metabolism of AA leads to generation of oxygen-derived free radicals [3]. Incubation of astrocytes from primary culture with AA demonstrated enhanced production of superoxide anions [3], potentially damaging to neurons and glial cells. In this study, preincubation of C6 glioma cells with SOD resulted in a significant reduction of AA-induced cell swelling (Fig. 2), demonstrating the involvement of superoxide anions in this process. The incomplete reduction of cell swelling by SOD might be explained by the polar structure of the molecule, reaching and scavenging only in part intracellular generated oxygen radicals. Free oxygen radicals may be generated not only via cyclo- and lipoxygenases but also via other mechanisms, such as cytochrome P 450 and AA autooxidation. Inhibition of cyclo- and lipoxygenases by BW 755C therefore might prove insufficient to prevent formation of oyxgen radicals.

Another important aspect of AA-induced glial swelling is the inhibition of the respiratory chain by AA, resulting in subsequent activation of anaerobic glycolysis with production of lactate [19]. Recent results have shown that addition of AA to glial cells led to an initial dose-dependent intracellular acidosis, which was reversed at a later stage during the experiment. This might be due to activation of the Na^+/H^+-antiporter, exchanging intracellular protons against extracellular Na^+ ions, thereby not only restoring the intracellular pH but also contributing to cell swelling via uptake of Na^+ ions [19]. The activity of the Na^+/H^+-antiporter can be reduced either by direct inhibition with amiloride [13] or indirectly by inhibition of PKC [7], a potent activator of the Na^+/H^+-antiporter [17], both leading to attenuation of AA-induced cell swelling (Fig. 2).

The most pronounced inhibition of AA-induced cell swelling was demonstrated by ouabain, an inhibitor of the Na^+/K^+-ATPase [13]. Other experiments performed in Na^+-free suspension medium showed an almost complete inhibition of glial swelling [19], confirming the importance of Na^+ ions in the swelling process. AA might increase intracellular Na^+ concentration via three different mechanisms, including activation of the Na^+/H^+-antiporter (see above), and initiation of lipid peroxidation, leading to disruption of ion channels and thus enhancement of cellular influx of Na^+ ions [3,18]. In addition, recent results have demonstrated that AA is taken up by C6 glioma cells, probably via a Na^+ co-transport system [20]. Inhibition of the Na^+/K^+-ATPase with subsequent breakdown of the Na^+ gradient should disturb all three mechanisms of Na^+ influx and therefore abolish AA-induced cell swelling (Fig. 3).

In summary, our results suggest a role for free radicals, signal transduction systems and intracellular accumulation of Na^+ ions in glial cell swelling from AA, whereas there is no evidence for the involvement of prostaglandins and leukotrienes in the swelling process.

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